IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: HADLACZKY *et al.*
Serial No. 09/724,872
Filed: November 28, 2000
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For: *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES*
Art Unit: 1638
Examiner: Helmer, G.L.

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ATTACHMENTS TO AMENDMENT

(1) Executed Declaration of Steven F. Fabijanski Pursuant to 37 C.F.R. §1.132

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DECLARATION PURSUANT TO 37 C.F.R. §1.132

Sir:

I, Steven F. Fabijanski, declare as follows:

1) I am familiar with the subject matter of the above-captioned application, which was filed on November 28, 2000.

2) I received a Bachelor's degree in Biology from the University of Miami (Florida) in 1977. I received a Ph.D. degree in Cellular and Molecular Biology from the University of Southern California in 1981. I have held post-doctoral positions at the University of Ottawa in Ottawa, Ontario, Canada and the University of Southern California in Los Angeles, California from 1982 to 1985. From 1986 to 1991, I held the position of Research Director at Paladin Hybrids, Inc.

3) I have over 20 years of experience in the areas of plant molecular biology, plant gene expression, plant tissue and cell culture and development of techniques to produce genetically modified plants and plant artificial chromosomes. I have authored or co-authored over 20 publications and I am an inventor of 15 US and foreign patents.

4) I am currently Research Director at Agrisoma Biosciences Inc., located in Saskatoon, Saskatchewan, Canada. Chromos Molecular Systems, Inc., located at 8081 Lougheed Highway, Burnaby, B.C., Canada V5A 1W9, is an owner of Agrisoma Biosciences, Inc. I have held this position since 2001. I am also President of FAAR Biotechnology Group Inc., located at Suite 323, 5929L Jeanne D'arc Boulevard, Orleans, Ontario, Canada K1C 7K2. I have held this position since 1992.

5) In my capacity as researcher, myself, persons under my direction and other research groups: the Scottish Crop Research Institute in Scotland; the Danforth Plant Science Center in St. Louis, Missouri; the Hungarian Biological Research Center in Hungary; and Applicant's research group at the Plant Biotechnology Institute in Saskatoon, Saskatchewan, Canada; have studied the introduction of satellite artificial chromosomes into plant cells and the generation of plant artificial chromosomes.

6) Using methods and materials described in the above-referenced application and standard methods as described herein, myself and other scientists involved in these projects have demonstrated that satellite artificial chromosomes can be transferred to plant protoplasts using either (a) microcell-mediated fusion of satellite artificial chromosome-containing murine cells with plant protoplasts or (b) lipid-mediated transfection of isolated satellite artificial chromosomes into plant protoplasts. Further, we have demonstrated that plant artificial chromosomes can be generated by introduction of heterologous DNA into plant cells and growth under selective conditions to produce cells containing plant satellite artificial chromosomes.

As exemplified by the results shown below, we have demonstrated element-for-element and step-for-step that, by following the teachings in the application, a satellite artificial chromosome can be introduced into a plant cell. In addition, as exemplified by the results shown below, we have demonstrated element-for-element and step-for-step that, by following the teachings in the application, plant artificial chromosomes can be generated by i) introducing a DNA fragment with a selectable marker into a plant cell; ii) growing the cell under selective conditions to produce plant cells that have incorporated the DNA into their genomic DNA such that a plant satellite artificial chromosome is produced; and iii) selecting a cell that contains a plant satellite artificial chromosome.

MATERIALS AND METHODS

A. Transfer of satellite artificial chromosomes into Plant Protoplasts

1. Transfer of satellite artificial chromosomes into Tobacco Cells

Satellite artificial chromosomes were introduced into tobacco cells using microcell-mediated fusion. Microcells were prepared from murine cells containing a satellite artificial chromosome as described in U.S. Patent No. 6,077,697 (of which this application is a continuation-in-part). Briefly, 5 x 10^6 EC3/7C5 cells (a mouse cell line deposited by Applicant in accord with the Budapest Treaty at the European Collection of Animal cell Culture (ECACC) under Accession No. 96040925) in a T25 flask were treated first with 0.05 µg/ml colcemid for 48 hr and then with 10 µg/ml cytochalasin B for 30 min. The T25 flask was centrifuged on edge and the pelleted microcells were suspended in serum free DME medium. The microcells were filtered through first a 5 micron and then a 3 micron polycarbonate filter, treated with 50 µg/ml of phytohemagglutin, and used for polyethylene glycol mediated fusion with recipient cells.

The microcells containing satellite artificial chromosomes were then fused with freshly prepared tobacco BY-2 protoplasts in a ratio of 10:1 (microcells to protoplasts). Fusion occurred in the presence of 20% PEG 4000 and 100-200 mM calcium chloride. The fusion and transfer of the chromosomes to the tobacco protoplasts was visualized with 4',6-diamidino-2-phenylindole•2HCl (DAPI) staining of the microcells by preincubation of the microcells with DAPI at a final concentration of 1 µg/ml. The fused protoplasts were recovered and allowed to grow for one or more generations.

To further demonstrate the transfer of mouse chromosomal sequences to tobacco protoplasts, the fused tobacco cells or nuclei isolated from fused tobacco cells were subjected to fluorescence *in situ* hybridization (FISH) analysis using biotin-labelled mouse major satellite DNA as a probe. To isolate nuclei from the fused tobacco cells, protoplast calli were digested with 1.2% Cellulase 'Onozuka' R-10 and 0.4% w/v Macerozyme R-10 in nuclei isolation buffer (10 mM MES pH 5.5, 0.2 M sucrose, 2.5 mM EDTA, 2.5 mM DTT, 0.1 mM spermine, 10 mM NaCl, 10 mM KCl and 0.15% Triton X-100) for 3 hours. After centrifugation at 80 x g for 10 minutes, the pellets of protoplasts were resuspended in hypertonic buffer of 12.5%

W5 solution (Hinnisdaels *et al.* (1994) *Plant Molecular Biology Manual G2*:1-13, Kluwer Academic Publisher, Belgium) for 10 minutes. To promote disruption of protoplasts, the protoplast suspension was forced through a syringe needle four times. The disrupted protoplasts were filtered through 5 µm meshes to remove debris and centrifuged at 200 x g for 10 min. By repeated washing of the pellet in a nuclei isolation buffer containing phenylmethylsulfonylfluoride (PMSF) and centrifugation at 200 x g for 10 minutes, nuclei were collected as a white pellet freed from cytoplasm contamination and cellular debris. Samples were fixed in 3:1 methanol:glacial acetic acid for FISH analysis.

FISH analysis to screen for the transfer of artificial chromosomes into plant cells was performed using DNA probes specific for the mouse major satellite DNA as described (Fransz *et al.*, *The Plant Journal, 9*: 421-430, 1996).

2. Transfer of satellite artificial chromosomes into *Arabidopsis* Cells

Satellite artificial chromosomes were introduced into *Arabidopsis* cells using microcell-mediated fusion. Microcells were prepared as described above. The prepared microcells were then fused with freshly prepared *Arabidopsis* protoplasts in a ratio of 10:1 (microcells to protoplasts). Fusion occurred in the presence of 25% PEG 6000, 204 mM $CaCl_2$, pH 6.9 within the first 5 minutes of mixing. Typically, less than about one minute of mixing was required to observe fusion between microcells and protoplasts. Fused cells were washed with 240 mM $CaCl_2$, then floated on top of a solution of 204 mM sucrose in B5 salts. Cells were then transferred to cell suspension culture media (MS, 87 mM sucrose, 2.7 µM napthalene acetic acid, 0.23 µM kinetin, pH 5.8).

Fused protoplasts were recovered and allowed to grow for one or more generations. Southern hybridization and PCR analysis using satellite sequences in the satellite artificial chromosome were used to detect the presence of satellite artificial chromosomes in the fused protoplasts. To further demonstrate the transfer of mouse chromosomal sequence to *Arabidopsis* protoplasts, *Arabidopsis* plant cell nuclei were isolated and subjected to FISH analysis using biotin-labelled mouse major satellite DNA as a probe, as described above.

3. Transfer of satellite artificial chromosomes into Rice Protoplasts

Isolated murine artificial chromosomes (MACs) prepared by sorting through a FACS apparatus and purified as described, *e.g.*, in U.S. Patent No. 6,077,697 (of which this application is a continuation-in-part), were transferred into rice plant protoplasts by cationic lipid- mediated transfection. First, 1 x 10^6 purified artificial chromosomes in liquid buffer were mixed with 15 µl of LipofectAMINE 2000 (Gibco, Md, USA). The satellite artificial chromosome/LipofectAMINE solution was allowed to complex for three hours, and then was added to a mixture of 1 X 10^5 freshly prepared rice protoplasts.

The uptake of the lipid-complexed artificial chromosome was monitored by adding a fluorescent dye that stains DNA to the mixture of protoplasts and purified artificial chromosomes. Microscopic examination of the protoplast/artificial chromosome mixture over the next several hours allowed the visualization of the artificial chromosome as it was transported across the protoplast cellular membrane and the presence of the readily identifiable MAC in the cytoplasm of the rice plant cell.

B. Generation of Plant Artificial Chromosomes (Plant SATACs)

1. Construction of heterologous DNAs

Vector pAgIIa, containing two selectable markers and a sequence with homology to the pericentric DNA, was constructed using standard techniques of molecular biology. A hygromycin phosphotransferase (HPT) gene under the control of the 35S promoter (see, for example, Blochinger *et al. Mol. Cell. Biol. 4*:2929-2931) was incorporated into the vector for selection. A 334 base pair sequence with homology to tobacco pericentric sequences (Genbank Accession No. Y08422, submitted 1996; see also Genbank Accession Nos. X76056 and D76443 submitted 1993 and 1995, respectively) was constructed, containing the central AT-rich region of a tobacco rDNA intergenic spacer capable of amplification (Borisjuk *et al.* (1997) *Plant Mol. Biol. 35*:655-660). Vector pAGIIa also contains a visible marker, constructed by placing a β-glucuronidase (GUS) gene under the control of the nos promoter (Novel *et al.* (1973) *Mol. Gen. Genet.* 120:319-335; Jefferson *et al.* (1986)

Proc. Natl. Acad. Sci. USA 83:8447-8451; US Patent No. 5,268,463; commercially available from Clontech Laboratories, Palo Alto, CA) and a detection marker containing a 234 base pair mouse major satellite DNA sequence derived from pSAT-1 (Wong *et al.* (1988) *Nucleic Acid Research, 16(24)*:11645-11661. The vector also contains a second selectable marker constructed from a phosphinothricin acetyl transferase (PAT) gene under the control of the 35S promoter, (see, for example, White *et al.* (1990) *Nuc. Acids Res. 18*:1062; Spencer *et al.* (1990) *Theor. Appl. Genet. 79*:625-631; Vickers *et al.* (1996) *Plant Mol. Biol. Reporter 14*:363-368; and Thompson *et al.* (1987) *EMBO J. 6*:2519-2523).

A targeting DNA was constructed with homology to pericentric DNA sequences. The targeting DNA contains a 1.7 Kb portion of the 26S rDNA coding region (Genbank accession X52320). The targeting DNA was cloned into the vector pBluescript (Stratagene, La Jolla, CA).

2. Introduction of DNAs into plant cells and selection

Vector DNA and targeting DNA were introduced into tobacco cells using PEG mediated transfection. Briefly, tobacco protoplasts were isolated from established sterile tobacco plant cultures by immersion of sterile tissue in enzyme solution containing 1.2% Cellulase 'Onozuka' R-10 and 0.4% Macerozyme R-10. The protoplasts were purified by pouring through a 100 µm nylon mesh sieve, overlaid with washing solution and centrifuged at 80 x g for 10 min. Protoplasts were then resuspended at a density of 1 $x10^6$ protoplasts/ml and stored at 4°C for 1 to 2 hours prior to DNA uptake.

The vector and targeting DNAs were sterilized with chloroform and 70% ethanol before use. A protoplast suspension was mixed with vector and targeting DNA at a ratio of 1:10, followed immediately by slow addition of a polyethylene glycol (PEG) solution. As controls, salmon sperm or calf thymus DNA was added instead of the targeting DNA. The mixture was incubated at 22°C for 10-15 min, with gentle shaking. The protoplasts were resuspended and cultured at 22°C in the dark. When microcalli developed, the protoplasts were embedded in 0.6% agarose. Selection on protoplast cultures was carried out by adding hygromycin to the medium at a final concentration of 20 mg/l, 14 to 21 days after transfection.

Calli that grew on selection were cultured under selective conditions for a period of 3 - 6 months, with frequent subculturing. Standard molecular biology techniques were used to verify the presence of the vector DNA.

3. Identification of amplified DNAs

GUS-expressing calli produced using vector DNA and either the targeting DNA or control DNA were subjected to two-color Fluorescent *In Situ* Hybridization (FISH) using two probes. The first probe was tagged with rhodamine (red fluorescence) and recognized pericentric DNA (18S rDNA) sequences endogenous to tobacco cells. The second probe recognized the detection marker (mouse major satellite sequence) in the pAgIIa vector used for transfection and was visualized with a fluorescein isothiocyanate (FITC) tag (blue-green fluorescence).

To obtain spreads of metaphase chromosomes, cells were subjected to either a single blocking protocol (colchicine treatment), or double blocking protocol (for example, treating plant cells with 5 mg/L aphidicolin for 24 hours and then 1.54 mg/ml Propyzamide for 4 hours). The blocked cells were recovered and chromosome spreads prepared and subjected to two-color FISH. Red and blue-green fluorescence was monitored to identify amplification. In general, 8-10 chromosome spreads were screened per sample. Further fluorescent image analysis was performed in a subset of the samples to overlay the probe signals and further detail chromosome structure.

RESULTS

The results provide evidence of satellite artificial chromosome delivery to plant cells by microcell-mediated fusion or lipid-mediated transfection. The results further demonstrate that transferred satellite artificial chromosome materials are detected in plant cells for at least 16 weeks following such transfer. The results also demonstrate that plant artificial chromosomes (plant SATACs) can be generated and maintained in plant cells.

A. Transfer of Satellite Artificial Chromosomes into Plant Protoplasts

In tobacco cells, protoplast fusion and artificial chromosome transfer were confirmed by DAPI staining. In addition, FISH analysis of either the cells or nuclei isolated from tobacco protoplasts fused with satellite artificial chromosome-

containing murine cells showed numerous cells and nuclei, respectively, that had incorporated mouse chromosomal nucleic acid. The signal was consistent with the ability to transfer and maintain an intact mouse satellite artificial chromosome in tobacco cells.

In *Arabidopsis* cells, Southern hybridization and PCR analysis demonstrated the presence of satellite artificial chromosome-related sequences in fused *Arabidopsis* protoplasts. In addition, FISH analysis demonstrated that a portion of the nuclei isolated following protoplast fusion contained mouse major satellite DNA. These results indicated successful transfer of one or more mouse chromosomes to the *Arabidopsis* nuclei. Further, PCR data indicated that satellite artificial chromosome material was still present in *Arabidopsis* protoplasts after 8 weeks of culture. In addition, FISH analysis undertaken on samples from the same *Arabidopsis* cultures 16 weeks post-fusion demonstrated the presence of satellite artificial chromosome material.

In rice protoplasts, isolated mammalian satellite artificial chromosomes were delivered by lipid-mediated transfection. Fluorescent dye analysis demonstrated incorporation of the purified artificial chromosome into rice protoplasts.

In all cases, microscopic evidence of the uptake of satellite artificial chromosomes into plant cells and FISH analysis of plant cells or nuclei revealed the presence of satellite artificial chromosome material in the plant cells within 24 hours of culture, and at rates of 1-5%.

B. Generation of Plant Artificial Chromosomes (Plant SATACs)

Following introduction of the heterologous DNAs into tobacco cells, cells were selected on hygromycin. More than 400 calli were obtained. A portion of the calli were analyzed for expression of the GUS reporter gene. A total of 31 independent GUS-expressing calli obtained using the targeting DNA or control DNAs were selected for further analysis.

The calli were subjected to two-color FISH using the probes for endogenous pericentric (18S rDNA) sequences and for the detection marker in the vector. The endogenous pericentric (rDNA) loci on tobacco chromosomes stained red, and regions of the chromosome where the vector DNA inserted stained blue-green. Where amplification of the vector DNA had occurred, medium to high blue-green

signal was observed. A low signal blue-green signal indicated a lack of large scale amplification of the vector sequence. Twenty-four calli produced with the vector and targeting DNA were analyzed for blue-green signal level: 5 were categorized as a medium level signal, 1 as high and 1 as medium high. The remaining 17 samples produced a low blue-green signal. The control calli, produced with vector DNA and either salmon sperm or calf thymus control DNAs, all produced low levels of blue-green signal. The probe for 18S rDNA recognizes the native pericentric sequences (rDNA) found in the tobacco chromosomes and thus should recognize all samples equivalently. This probe provided a red signal of high intensity in all of the analyzed samples.

Thus, in 7 out of 24 analyzed calli produced with targeting DNA homologous to pericentric sequences, large scale amplification of the vector sequences was observed at the chromosome level. No such amplification was observed using salmon sperm or calf thymus DNAs, indicating that targeting DNA without known homology to pericentric DNA is less efficient for stimulating large scale amplification.

Overlap analysis of the probes was used to further determine the fate of targeting and vector DNAs. Overlap of blue-green and red signals indicated that homologous recombination of the targeting DNA and vector DNA had occurred. Additionally, areas where significant levels of both blue-green and red signals were observed, demonstrated large scale amplification of pericentric regions.

One of the 7 callus lines with a medium-high vector signal was analyzed further. This line was shown to contain a chromosome that exhibited large scale "sausage" amplification and a breakage product representing a plant artificial chromosome (plant SATAC). Comparison of the rhodamine and FITC signals demonstrated that the vector was inserted and amplified in two of the eight visible pericentric (rDNA) loci. The first locus was identified as a "sausage" amplification by the tandemly duplicated nature of the vector and pericentric DNA signals. This locus represented a formerly dicentric chromosome and/or a sausage chromosome. The second locus identified a breakage product, indicating the creation of a plant SATAC by breakage of a formerly dicentric chromosome. The plant SATAC contained vector sequence integrated into plant pericentric satellite DNA.

The SATAC was clearly visible in chromosome spreads as a small independent chromosome entity containing both amplified vector DNA and pericentric DNA. Image analysis was used to overlay both pericentric DNA and vector DNA signals. In high resolution images, the presence of both amplified vector (blue-green signal) and pericentric heterochromatic DNA (red signal) was repeatedly observed. Metaphase images of the SATAC demonstrated the presence of small chromosome arms and a constriction representing the centromere region. Because amplified DNA sequences were detected with the probe specific for pericentric sequences (18S rDNA), it represented newly amplified DNA. This signal can not be attributed to the introduced targeting DNA used, because the targeting DNA contained only 26S rDNA sequences.

Thus, this callus line demonstrated the production of two amplified regions as a result of insertion of vector DNA into the pericentric DNA. The analysis indicated targeting of the vector DNA to pericentric DNA and evidence for large scale amplification, including "sausage" amplification. A SATAC, produced from a breakage product, was identified that contained amplified vector DNA as well as heterochromatic DNA. The callus line containing the plant SATAC was stably maintained in culture for over 6 months.

CONCLUSION

The above experiments demonstrate that satellite artificial chromosomes can be transferred to plant cells. Evidence of satellite artificial chromosome transfer to plant cells can be detected within 24 h and for up to at least 16 weeks following such transfer. Thus, by following the teachings of the specification and standard methods as described herein, one can introduce satellite artificial chromosomes into plant cells.

The above experiments further demonstrate that a plant artificial chromosome (plant SATAC) can be generated by introducing heterologous DNA into a plant cell, growing cells under selective conditions to produce cells that have incorporated the DNA such that a plant satellite artificial chromosome is produced and selecting a cell that contains a plant SATAC. The plant SATAC contains a plant centromere, as well as amplified pericentric DNA and the introduced heterologous DNA. The plant

SATAC can be detected in cells and the cells can be maintained in culture for over 6 months.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

March 03 2004
Date

Steven P. Fabijanski

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